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HPTLC METHOD DEVELOPMENT AND VALIDATION OF A SECONDARY METABOLITE – β - SITOSTEROL FROM *CAESALPINIA BONDUC* (LINN.) ROXB. EMEND. DANDY & EXELL. SEEDS

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ABSTRACT:

Caesalpinia bonduc (Linn.). is commonly called as latakaranj and is widely distributed throughout India. A sensitive, simple, and accurate High-Performance Thin-Layer Chromatographic method has been established for quantitation of β-sitosterol from seed powder of *Caesalpinia bonduc* (Linn.) collected from different regions of India as well as from Menstrowin tablets, a polyherbal formulation used against female reproductive disorder. The seed powder was extracted with Methanol and used for quantitation. The concentration of β-sitosterol was found to be 0.1134 mg/gm. in the seed powder of *Caesalpinia bonduc* (Linn.) collected from Mumbai which was used as a reference sample. Quantitation of β-sitosterol was also carried out for *Caesalpinia bonduc* (Linn.) seeds collected from different regions of India like Kutch (Gujrat), Malvan, Madhya Pradesh and Raigad and the concentrations were found to be 0.2080 mg/gm, 0.1432 mg/gm, 0.1244 mg/gm and 0.0820 mg/gm respectively. The concentration of β-sitosterol from polyherbal formulation Menstrowin (Safe Life Herbals Pvt, ltd.) was found to be 0.02 mg/gm. Quantitation was carried out on HPTLC silica gel 60 F_{254} pre-coated plates with the mobile phase Toluene: Ethyl Acetate: Methanol (7:1:0.5) (v/v/v). A TLC scanner set at 366nm in fluorescence **/** reflectance mode was used for quantitation. β-sitosterol response was linear over the range 5 µg mL^{-1} to 50 µg mL^{-1}. The method was validated for linearity, precision, accuracy and robustness.

KEY WORDS:

Caesalpinia bonduc (Linn.) Roxb. emend Dandy & Exell. seeds, HPTLC, β-sitosterol and Quantitation.

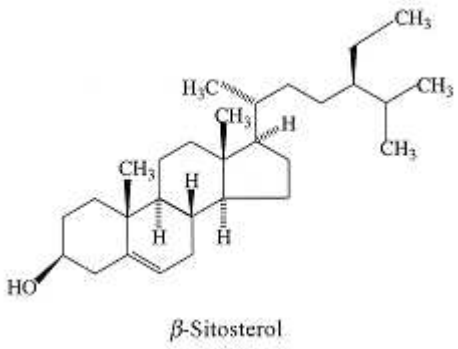
1. INTRODUCTION:

Caesalpinia bonduc (Linn.) belongs to family Leguminosae and Sub family: Caesalpinaceae. It is commonly called as Latakaranj and is widely distributed throughout India, in Himalayas upto 1000m, in hotter parts, also found in the plains on waste lands and

coastal areas. It is common in Bengal and South India [1] .The seeds of *Caesalpinia bonduc* (Linn.) are reported to have many therapeutic uses like anti-diabetic, anti-fertility and anti-estrogenic activity [2] .Several herbal industries have been using *Caesalpinia bonduc* (Linn.) seeds in herbal formulations which are used in managing menstrual disorders and Uterine disorders, e.g. Menstrowin tablets. The seeds of *Caesalpinia bonduc* (Linn.) contain fatty acids like Palmitic acid, Stearic acid, Oleic acid, a non crystalline bitter glycoside Bonducin . It also contains Phytosterins, Caesalpins, Bonducellin and Citrullin. The kernel contains fatty oil, starch, sucrose two phytosterols one of them is identified as sitosterol like ß-sitosterol, a secondary metabolite, which is a bioactive compound[3]. ß-sitosterol, is reported to help in curing Hyperlipidemia, Cholesterol absorption, Breast Cancer, Immunomodulator, Aging Prostrate gland and Gynecological disorders[4,5]. The structure of β-sitosterol is shown in Figure [1]

[Figure 1]

Structure of β-sitosterol standard [12]



β-Sitosterol

The literature reveals that there is no High-Performance Thin-Layer Chromatographic method available for quantitation of β-sitosterol from seeds of *Caesalpinia bonduc* (Linn.), however quantitation of β-sitosterol has been done from other plants like *Cynodon dactylon* (Linn.) Pers. [6], *Asteracantha longifolia* Nees [7], *Woodfordia fruiticosa* (Linn.) Kurz [8] etc. In the present research, a sensitive, simple, and accurate High-Performance Thin-Layer Chromatographic method has been established for quantitation of β-sitosterol from *Caesalpinia bonduc* (Linn.) seeds collected from different regions of India and a polyherbal formulation (Menstrowin) containing *Caesalpinia bonduc* (Linn.) seeds .

2. EXPERIMENTAL:

2.1. MATERIALS:

Caesalpinia bonduc (Linn.) seeds were collected from Mumbai (Maharashtra) and was authenticated from Agharkar Institute (Pune)-Auth 08-68. Standard β-sitosterol (98% purity) was procured from Sigma Aldrich Chemie

(Steinheim, Germany). The solvents Toluene, Ethyl Acetate and Methanol were of analytical grade and were purchased from Qualigens Fine Chemicals, Mumbai, India, were used for the analysis.

2.2. INSTRUMENTS:

A TLC scanner Camag 2 with a computer system and Cats 3 Version Software (Camag, Muttenz, Switzerland) was used. The source of radiation was Mercury lamp. Camag Linomat IV was used as applicator. Separation was done on HPTLC silica gel 60 F_{254} pre-coated plates procured from Merck (Darmstadt, Germany).

2.3. STANDARD AND SAMPLE PREPARATION:

A stock solution of β-sitosterol (1000 µg mL^{-1}) was prepared by dissolving 10.0 mg of accurately weighed β-sitosterol in Methanol and diluting it to 10.0 mL with Methanol. Aliquots (0.05 mL to 0.5 mL) of this stock solution were transferred to 10 mL standard volumetric flasks and the volume of each was adjusted to 10 mL with Methanol, to obtain working standards containing 0.5 µg mL^{-1} to 50 µg mL^{-1}.

Seeds of *Caesalpinia bonduc* (Linn.) were collected, washed, shade dried, powdered, sieved through an 80 mesh (BSS) sieve and stored in an airtight container at 25°C. 1.0 gm of the dried powder was accurately weighed, placed in a stoppered tube and 10 mL of Methanol was added. The sample was vortexed for 1-2 min and left to stand overnight at room temperature (28 ± 2°C). The contents of the tube were filtered through Whatmann filter paper No. 41 (E. Merck, Mumbai, India). The clear supernatant was collected and used for Quantitation and Validation. Menstrowin tablets were procured from local market. Five tablets were weighed accurately and crushed to obtain a fine powder. The weight equivalent to one tablet was determined and 1.0 g powder was then transferred in a clean, dry, stoppered volumetric flask and 10 mL Methanol was added. The solution was vortexed for 1-2 min, allowed to stand overnight and filtered through Whatmann filter paper No. 41. This solution was used for further analysis.

2.4. CHROMATOGRAPHY: [10, 11]

2.4.1. PROCEDURE:

Chromatography was performed on HPTLC silica gel 60 F_{254} pre-coated plates. Samples (10 µL) were applied on the plates as bands of 10 mm width with the help of a Camag Linomat IV sample applicator at the distance of 15mm from the edge of the plates. The mobile phase constituted of Toluene- Ethyl Acetate- Methanol 7.0+1.0+0.5 (*v/v/v*). The plates were developed up to a distance of 85 mm in a Camag twin-trough chamber previously equilibrated with mobile phase for 30 min. The chromatographic conditions had previously been optimized to achieve the best resolution and peak shape. After development, plates were dried under current of air at room temperature, derivatised with freshly prepared 10 % Methanolic Sulphuric acid reagent in a derivatisation chamber for 20 secs, and dried at room temperature. After drying, plates were heated in oven at 105°C for 10 mins before densitometric scanning [9]. Densitometric evaluation of the plates was performed at 366 nm in fluorescence/reflectance mode using Mercury lamp with a Camag Scanner II in conjunction with Cats 3 Version Software. The chromatographic plates of (i) *Caesalpinia bonduc* (Linn.) seeds collected from Mumbai (Reference sample) and β-sitosterol, (ii) *Caesalpinia bonduc* (Linn.) seeds collected from different regions like Madhya Pradesh , Kutch (Gujrat), Raigad, Malvan and Mumbai and β-sitosterol standard, (iii) Formulation (Menstrowin)

containing *Caesalpinia bonduc* (Linn.) seeds and β-sitosterol standard are shown in **Plate 1, 2, 3 respectively.** Their respective HPTLC chromatograms are shown in **Figure 1a, 2a, and 3a respectively.**

Plate1

Plate photo of *Caesalpinia bonduc* (Linn.) seeds and β-sitosterol (after derivatization) in fluorescence (366 nm)

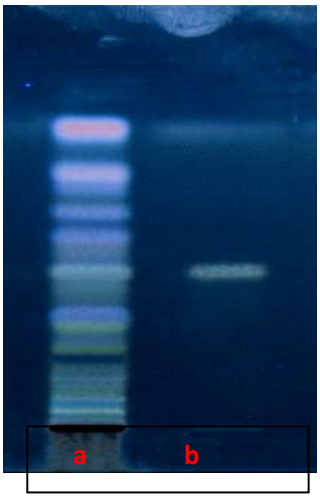
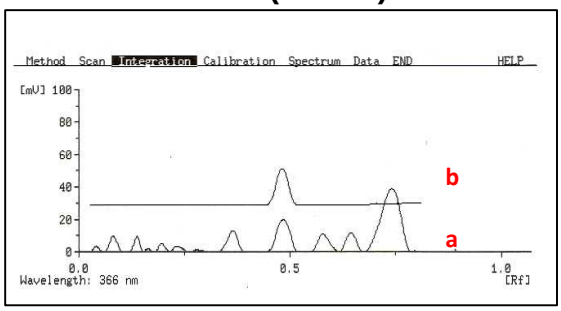


Figure 1.a

An overlay of densitometric scan of seeds of *Caesalpinia bonduc* (Linn.) and β -sitosterol in fluorescence (366nm)



Track a: Seeds of Caesalpinia bonduc (Linn.) collected from Mumbai (Reference sample).
Track b: β sitosterol standard (20ppm)

a

Plate 2

Plate photo of *Caesalpinia bonduc* (Linn.) seeds collected from different regions and β-sitosterol (after derivatization) in fluorescence (366nm).

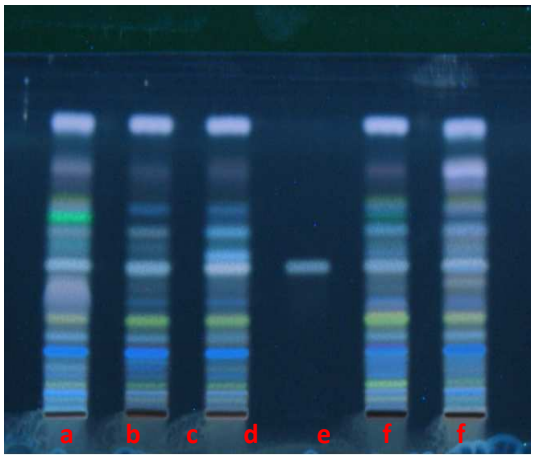
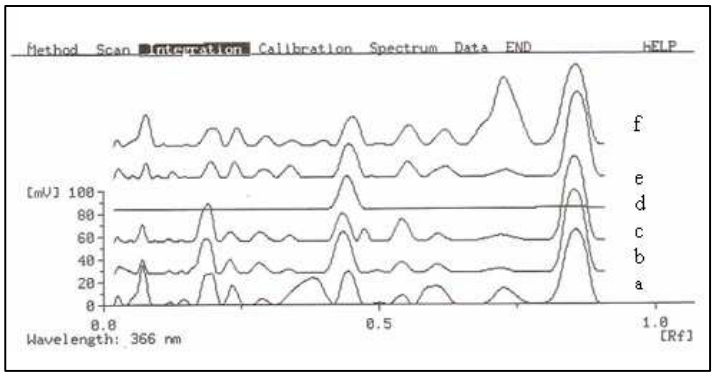


Figure 2.a

An overlay of densitometric scan of seeds of *Caesalpinia bonduc* (Linn.) collected from different regions and β-sitosterol in fluorescence (366nm).



Track a: Madhya Pradesh
Track b: Kutch (Gujrat)
Track c: Raigad
Track d: β sitosterol (20 ppm).
Track e: Malvan
Track f: Mumbai

Plate 3

Plate photo of *Caesalpinia bonduc* (Linn.) seeds, β-sitosterol and polyherbal formulation (Menstrowin) (after derivatization) in fluorescence (366nm).

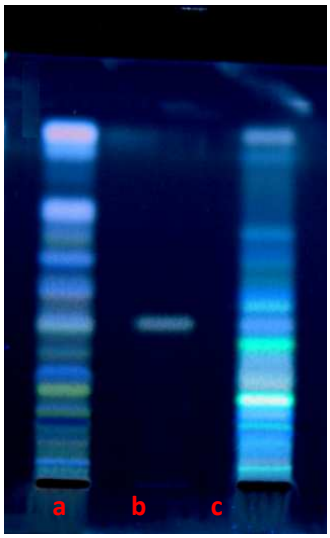
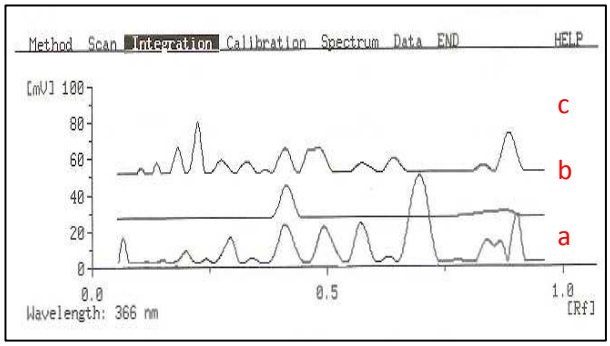


Figure 3.a

An overlay of densitometric scan of seeds of *Caesalpinia bonduc* (Linn.), β-sitosterol and polyherbal formulation (Menstrowin) in fluorescence (366nm).



Track a: Seeds of Caesalpinia bonduc (Linn.)
Track b: β sitosterol (20 ppm)
Track c: Menstrowin

2.4.2. LINEARITY OF DETECTOR RESPONSE:

Solutions containing β-sitosterol at ten different concentrations (5, 10, 15, 20, 25, 30, 35, 40,45, 50 µg mL^{-1}) were prepared in Methanol. Each of these solutions (10 µL) was applied on a plate, the plate was developed and the detector response for different concentrations was measured. A graph was plotted using the peak area against concentration of β-sitosterol. The plot was linear in the range 5 to 50 µg mL^{-1}. The experiment was performed three times and the mean was used for the calculations. The linearity data is given in **Table 1.**

Table 1

Linearity Data.

Linearity range	5 to 50 µg mL^{-1}
Slope (m)	34.588
Intercept(c)	231.35
Correlation coefficient (R)	0.9991
LOD	0.5 µg mL^{-1}
LOQ	1 µg mL^{-1}
System Suitability (n =5 %CV)	0.09
Instrument Precision (n=6 %CV)	0.11
Intraday (precision)(n=3 %CV)	0.06
Interday (precision)(n=3 %CV)	0.12

(y = mx + c, where, y= peak area, m= slope, x= concentration, c= intercept.)

2.4.3. ASSAY PROCEDURE:

The standard solution of β-sitosterol (20 µg mL^{-1}) and 10µL of sample solutions were spotted on a TLC silica gel 60 F_{254} pre-coated plates. The amount of β-sitosterol present in this solution was calculated by comparison of area measured for the sample to that for the standard. The assay procedure described earlier was repeated seven times. The results of assay are given in **Table 2**.

The amount of β-sitosterol was found to be in 0.2080 mg /gm, 0.1432 mg /gm, 0.1244 mg /gm, 0.1134 mg/gm, and 0.0820 mg/gm from *Caesalpinia bonduc* (Linn.) seeds collected from Kutch (Gujrat), Malvan, Madhya Pradesh, Mumbai, Raigad respectively and 0.02 mg/gm from polyherbal formulation (Menstrowin) containing seeds of *Caesalpinia bonduc* (Linn.) .

Table 2

Results of Assay

Sample Plant powder (seeds) of *Caesalpinia bonduc* (Linn.) seeds	Weight of sample in mg	Amount of β-sitosterol present in plant sample in mg/gm
Kutch (Gujrat)	1000	0.2080
Malvan	1000	0.1432
Madhya Pradesh	1000	0.1244
Mumbai	1000	0.1134
Raigad	1000	0.0820
Powder of Menstrowin tablets (polyherbal formulation)	1000	0.02

2.4.4. ACCURACY/RECOVERY:

The accuracy of the method was established by performing recovery experiments by the standard addition method. Recovery of standard β-sitosterol added to the extract of seed powder of *Caesalpinia bonduc* (Linn.) collected from Mumbai and powder of polyherbal formulation (Menstrowin) was studied at two different levels, each being analyzed in a manner similar to that described for the assay. The β- sitosterol content and the percent recovery was calculated. The results are given in **Table 3.**

Table 3
Results of Accuracy /Recovery Analysis

Amount of β-sitosterol in preanalysed sample of *Caesalpinia bonduc* (Linn.) seeds (mg/g)	Amount of β-sitosterol standard added to preanalysed sample of *Caesalpinia bonduc* (Linn.) seeds (mg/g)	Total amount of β-sitosterol (mg/g)	SD	% CV N=7	Recovery (%)
0.1134	0	0.1135	0.0002	0.2265	100.08
0.1134	0.028	0.1415	0.0002	0.1716	100.07
0.1134	0.056	0.1695	0.0003	0.2319	100.05
	Mean Recovery				**100.07**

3. RESULTS AND DISCUSSION:

In the current study, β-sitosterol was detected and quantified using HPTLC silica gel 60 F_{254} pre-coated plates with the mobile phase made of Toluene - Ethyl Acetate- Methanol 7.0 + 1.0 + 0.5 (*v/v/v*). The identity of the band of β-sitosterol in the plant extract was confirmed by overlaying the chromatogram of plant with that of the β-sitosterol standard and by comparing their R_F (0.42).

The detection of β-sitosterol was observed to be linear over a concentration range of 5 to 50 µg mL^{-1} with correlation coefficient of 0.9991. The concentration of β-sitosterol was found to be

0.1134 mg/gm in seeds of *Caesalpinia bonduc* (Linn.) collected from Mumbai. The concentration of β-sitosterol was found to be 0.2080 mg/gm, 0.1432 mg/gm, 0.1244 mg/gm, and 0.0820 mg/gm in seeds of *Caesalpinia bonduc* (Linn.) collected from Kutch (Gujrat), Malvan, Madhya Pradesh and Raigad respectively. β-sitosterol was also quantitated from a polyherbal formulation Menstrowin used against female reproductive dysfunction and was found to be 0.02 mg/gm.

System suitability, Instrument precision, intraday assay precision, interday assay precisions were measured to evaluate the precision of the method. The % CV values were found to be less than 2%, indicating that the selected method is precise and reproducible.

The accuracy of the method was established by means of recovery experiment. The mean recovery was close to 100%, which indicates that the method is efficient. The mean recovery of β-sitosterol was 100.07%.

The robustness of the method was studied, during method development, by determining the effects of small variation, of mobile phase composition (±2%), chamber saturation period, development distance and scanning time (10% variation of each). No significant change of R_f or response to β-sitosterol was observed, indicating the robustness of the method.

4. CONCLUSION:

The proposed method is simple, rapid, selective, sensitive, and economical and can be used for routine quality-control analysis and quantitation of β-sitosterol from seeds of *Caesalpinia bonduc* (Linn.) collected from different geographical regions of India as well from the formulation containing seeds of *Caesalpinia bonduc* (Linn.).

Maximum content of ß-sitosterol was found to be in seeds of *Caesalpinia bonduc* (Linn.) collected from Kutch (0.208mg/gm) while minimum content was found to be in seeds collected from Raigad (0.082 mg/gm).

Thus, ß-sitosterol can be used as a phytochemical marker to identify the raw material with maximum concentration of ß-sitosterol for the use in polyherbal formulation against female reproductive dysfunction. Since maximum concentration of ß-sitosterol was found to be in *Caesalpinia bonduc* (Linn.).seeds collected from Kutch it could play a vital role for the use in Ayurvedic companies manufacturing polyherbal formulations containing seeds of *Caesalpinia bonduc* (Linn.) .

REFERENCES:

1. Indian Council of Medical Research, Quality Standards of Indian Medicinal Plants Vol II, 25-33, (2005).
2. Ministry of Health and Family Welfare, API: The Ayurvedic pharmacopoeia of India, Government of India; New Delhi; Vol I, (2001).
3. Ali M.S., Shameel S., Ahmad V.U., Usmanghani K. , (1997). Chemical Constituents of *Caesalpinnia bonduc*. Pakistan Journal of Scientific and Industrial Research, ISSN :0030-9885:Vol 40 (1-4) Pg 20-22, (2007).
4. Becker M, Staab D, Von Bergmann K, Long-term treatment of severe familial hypercholesterolemia in children. Effect of sitosterol and bezatibrate. Pediatrics. 89:138-142,(1992).
5. Best MM, Duncan CH. Modification of abnormal serum lipid patterns in atherosclerosis by administration of sitosterol. *Ann Intern Med,* 45:614-622,(1956).

6. Champanerkar P. A., Vaidya V. V., Sunita Shailajan, Singh G. R. And Shah Willy. High Performance Thin- Layer Chromatographic method for quantification of β-sitosterol from *Cynodon dactylon*(Linn) Pers. India Drugs 44 (1) January , (2007).
7. S Sunita, S. Abhishek. A comparative evaluation of Phytochemical Fingerprints of *Asteracantha longifolia* Nees using HPTLC. Asian Journal of Plant Sciences, Vol 7th Issue no 6th, Pg 611-614,(2008).
8. Smruti Shah, Sunita Shailajan, Quantitation of β -Sitosterol from *Woodfordia fruiticosa* (Linn.)Kurz and a polyherbal formulation used for treating female Reproductive Disorder. An Indian Journal of Analytical Chemistry. Vol 8, Issue 1.Pg 82-86, (2009).
9. P. D. Sethi, High-Performance Thin Layer Chromatography. Quality Analysis of Pharmaceutical Formulations, CBS, India, (1996).
10. Rich E., Schibli A. , HPTLC for the Analysis of Medicinal Plants Thieme Medical Publisher, Inc, ISBN 13:978-1-58890-409-6(US), (2008).
11. Sherma and B.Fried, Handbook of Thin Layer Chromatography, (1996).
12. The Merck Index, 11th edition (1989), 8500.